RESEARCH ARTICLE

A Conserved Role for VEGF Signaling in Specification of Homologous Mesenchymal Cell Types Positioned at Spatially Distinct Developmental Addresses in Early Development of Sea Urchins

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ABSTRACT

Comparative studies of early development in echinoderms are revealing the tempo and mode of alterations to developmental gene regulatory networks and to the cell types they specify. In euechinoid sea urchins, skeletogenic mesenchyme (SM) ingresses prior to gastrulation at the vegetal pole and aligns into a ring-like array with two bilateral pockets of cells, the sites where spiculogenesis will later occur. In cidaroid sea urchins, the anciently diverged sister clade to euechinoid sea urchins, a homologous SM cell type ingresses later in development, after gastrulation has commenced, and consequently at a distinct developmental address. Thus, a heterochronic shift of ingression of the SM cell type occurred in one of the echinoid lineages. In euechinoids, specification and migration of SM are facilitated by vascular endothelial growth factor (VEGF) signaling. We describe spatiotemporal expression of *vegf* and *vegfr* and experimental manipulations targeting VEGF signaling in the cidaroid *Eucidaris tribuloides.* Spatially, *vegf* and *vegfr* mRNA localizes similarly as in euechinoids, suggesting conserved deployment in echinoids despite their spatially distinct development addresses of ingression. Inhibition of VEGF signaling in *E. tribuloides* suggests its role in SM specification is conserved in echinoids. Temporal discrepancies between the onset of *vegf* expression and SM ingression likely result in previous observations of SM "random wandering" behavior. Our results indicate that, although the SM cell type in echinoids ingresses into distinct developmental landscapes, it retains a signaling mechanism that restricts their

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spatial localization to a conserved developmental address where spiculogenesis later occurs. *J. Exp. Zool. (Mol. Dev. Evol.) 00B:1–10, 2017.*

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Pattern formation in embryogenesis relies on the precise spatial positioning of cell types and tissues. Cell types have recently been defined as the regulatory interactions and mechanisms that maintain conserved gene expression, or regulatory states, in any given lineage (Arendt et al., 2016). Migration of numerous cell types over short and long distances in the embryo ensures their proper spatial positioning at distinct developmental addresses, which we define here as their spatial orientation and location in the embryo. Migratory cells utilize external cues involving short- and long-range signaling to guide their course. An oft-utilized signaling pathway in cell motility and migration is vascular endothelial growth factor (VEGF) and its tyrosine kinase mediated receptors (Ferrara et al., 2003). VEGF signaling is required for numerous developmental processes in bilaterians (Kipryushina et al., 2015), including blood vessel formation and function (Ruhrberg et al., 2002; Gerhardt et al., 2003; Bautch, 2012), neural crest migration (McLennan et al., 2010), neurogenesis (Popovici et al., 2002), and cardiac development (Cleaver and Krieg, '98).

In sea urchins, migratory cells and ectodermal-mesenchymal signaling play an important role in pattern formation early in embryogenesis (Ettensohn, '90). The earliest migratory cell type in many echinoids is skeletogenic mesenchyme (SM), which ingresses into the blastocoel and migrates to two ventrolateral clusters where they synthesize spicules and, eventually, arms of the larval skeleton. The SM cell type is present in both sister clades of sea urchins, cidaroids and euechinoids, and evidence from molecular and developmental lineage analyses supports the homology of these cell types (Wray and McClay, '88). However, as was pointed out by the earliest comparative embryological observations (Tennent, '14), the timing and thus the developmental stage, or landscape, of mesenchymal ingression are different in these two sister clades. In euechinoids, SM ingresses into the blastocoel prior to gastrulation near the base of the vegetal pole, whereupon once within they migrate into a ring-like alignment that features two ventrolateral clusters where spicules are made (Gustafson and Wolpert, '61). In cidaroids, SM is loosely dispersed at the vegetal pole at the onset of gastrulation, ingresses at the tip of the archenteron only after the latter's extension well into the blastocoel, and subsequently migrates distally from the tip of the archenteron to the two ventrolateral clusters near the blastopore (Wray and McClay, '88; Yamazaki et al., 2014; Erkenbrack and Davidson, 2015). These descriptions suggest that the SM cell type has undergone heterochronic modifications in one of the two modern lineages of echinoids, and consequently each cell type finds itself at a different developmental address when it ingresses into the blastocoel, which poses nontrivial spatial orientation challenges since they eventually migrate to similar embryonic locations.

In euechinoids, proper positioning of SM into their ring-like alignment after their ingression into the blastocoel, as well as their specification, requires cues from VEGF and FGF signaling in the nearby ectoderm (Duloquin et al., 2007; Röttinger et al., 2008; Adomako-Ankomah and Ettensohn, 2013; McIntyre et al., 2014; Piacentino et al., 2016). In the euechinoid echinoids *Paracentrotus lividus, Hemicentrotus pulcherrimus, Strongylocentrotus purpuratus,* and *Lytechinus variegatus,* mRNA of *vegf* (also called *vegf3*) is spatially restricted proximal to the sites of spiculogenesis at two ventrolateral positions near the endodermal-ectodermal boundary, and mRNA of its receptor *vegfr* is expressed specifically in the SM lineage (Duloquin et al., 2007; Fujita et al., 2010; Li et al., 2012; Adomako-Ankomah and Ettensohn, 2013). Overexpression or perturbation of these spatial localization mechanisms results in aberrant skeletogenesis and absence of spicules (Duloquin et al., 2007; Adomako-Ankomah and Ettensohn, 2013; Sun and Ettensohn, 2014). Additionally, skeletogenic-specific regulatory genes, for example, *alx1,* have been shown to act upstream of *vegfr* in SM (Rafiq et al., 2012; Adomako-Ankomah and Ettensohn, 2013), and recombinant VEGF3 has been shown to be necessary for proper spicule formation in cell culture (Knapp et al., 2012). Taken together, these observations are in line with the proposed evolutionary co-option of the adult skeletogenic gene regulatory network (GRN) into early development of multiple lineages of echinoderms (Gao and Davidson, 2008) and are highly suggestive of a conserved ectodermal-mesenchymal GRN facilitating migration and specification of the SM cell type in euechinoids.

The preceding evidence begs the question as to whether the signaling mechanisms that specify and guide SM to the sites of spicule synthesis in early development of euechinoids are also functioning in their sister clade, the cidaroids, despite the fact that this homologous lineage ingresses into the blastocoel later in development and at a developmental address that is far removed from the future sites of spiculogenesis. A first clue was provided by a previous study that examined juveniles of the cidaroid echinoid *Eucidaris tribuloides* and implicated *vegfr* in

the positioning of juvenile skeletogenic structures by showing spatial restriction of its RNA proximal to expression of *alx1*, a regulatory gene involved in echinoid skeletogenesis (Erkenbrack and Davidson, 2015; Gao and Thompson et al., 2015). To date, no studies have investigated the spatiotemporal deployment or roles of VEGF and VEGFR in early development of the sister subclass cidaroid sea urchins. This is all the more interesting, as outgroup brittle star echinoderms, which also possess an early ingressing SM lineage as in euechinoids, utilize VEGF and VEGFR for localization and specification of SM both in early development (Morino et al., 2012), although this is not the case in early development of asteroids, another outgroup echinoderm that does not possess a larval skeleton (Morino et al., 2012). Therefore, among eleutherozoan echinoderms, cidaroids exhibit a developmental anomaly that has the potential to reveal how conserved homologous cell types cope with altered spatial confines while being tasked with the same developmental function.

To throw more light on this developmental evolutionary discussion, we reveal here the temporal expression and spatial distribution of *vegf* (known as *vegf3* in *S. purpuratus*) and *vegfr* (known as *vegfr-10-ig* in *S. purpuratus*) in early development of *E. tribuloides*. We find that, as in ophiuroids and euechinoids, *vegfr* is expressed specifically in the SM lineage and that *vegf* is spatially restricted to two ventrolaterally positioned regions of the embryo. To assay the role of VEGF signaling in *E. tribuloides*, we cultured embryos in the presence of the pharmacological VEGFR-inhibitor DMH4 (Hao et al., 2010). These data indicate that, although the SM lineage in cidaroids ingresses at a distinct development address and thus must traverse a distance of more than 50 μm (Wray and McClay, '88), VEGF signaling in *E. tribuloides* participates in specification of their homologous SM cell lineage. It is therefore highly probable that, despite the distinct spatial differences of ingression of SM in modern echinoids, VEGF signaling was utilized in the common ancestor of these clades and serves a critical function during skeletogenesis in eleutherozoan echinoderms. Lastly, we place our findings in a comparative evolutionary context within the echinoderm clade, as well as discuss the potential of echinoderm taxa for studying the developmental evolution of cell types and their underlying GRNs.

MATERIALS AND METHODS

Animals were acquired from KP Aquatics (Tavernier, FL). Embryos were cultured in controlled conditions at 22°C. Probe preparation, embryo fixation, whole-mount in situ hybridization (WMISH) and quantification of RNA transcript abundance by quantitative PCR (qPCR) were all conducted as previously described (Erkenbrack and Davidson, 2015; Erkenbrack, 2016; Erkenbrack et al., 2016). In *S. purpuratus*, there are three annotated VEGF ligands (VEGF, VEGF2, and VEGF3) and two annotated VEGF receptors (VEGFR-7-IG and VEGFR-10-IG) (Duloquin et al., 2007). *Eucidaris tribuloides* RNAseq transcriptomes that contain RNA transcripts from mixed developmental stages and that are annotated with *S. purpuratus* peptides revealed that *E. tribuloides* possesses orthologs for at least *Sp-vegf2* (SPU_005737), *Sp-vegf3* (SPU_030148), *Sp-vegfr-7-ig* (SPU_021021), and *Sp-vegfr-10-ig* (SPU_000310) (www.echinobase.org). We attempted to clone these four transcripts from cDNA libraries of early stage (0–72 hpf) *E. tribuloides* embryos and were able to obtain PCR amplicons for the orthologs of *Sp-vegf3* and *Sp-vegfr-10-ig*. In keeping with Duloquin et al. (2007), we refer to the ortholog of *Sp-vegf3* here simply as *vegf* and the ortholog of *Sp-vegfr-10-ig* as *vegfr*. Primers utilized during qPCR for *vegf* were forward 5′-GCTGTGGATAGTGAGAAGAAGG-3′ and reverse 5′-GGTAGTCGGACAGGTTGC-3′. For *vegfr* qPCR, primers were forward 5′-TCCTCATTGTGGTCATCTTACG-3′ and reverse 5′-AACTCCCACTTGGAATCATAGG-3′. For WMISH, probes were synthesized from plasmids that were produced by standard *E. coli* cloning of PCR fragments from cDNA libraries. PCR primers for WMISH probe cloning for *vegf* were forward 5′-CGTCGATTGATTGAGCAGAGA-3′ and reverse 5′-GCGAAAGAATAACGCCCTTTAC-3′. For *vegfr,* PCR primers were forward 5′-CACACCTTTACTGCAAACTGC-3′ and reverse 5′-AAACCTCCCATAAGAACACTCC-3′. Digoxygenin probes were synthesized following the manufacturer's protocol (T7 Transcription Kit; Roche, Mannheim, Germany). Partial sequences of cloned PCR products have been deposited at NCBI Genbank. Phylogenetic reconstruction was conducted on sequences obtained from cloning to confirm orthology within the ambulacraria (see Supplementary Material).

The vegfr-specific pharmacological inhibitor DMH4 (Tocris Biosciences, Ellisville, MO, USA, Cat. 4471) was reconstituted in dimethylsulfoxide (DMSO) to yield a stock concentration of 10 mM. Concentration and timing of treatment of *E. tribuloides* embryos was determined by dilution series. Embryos grown in the presence of 0.6% DMSO served as a control for DMSO toxicity. Optimal concentration for aberrant SM specification was found to be 3 μM, and timing of developmental exposure was found to be best after 15 hr of development. Embryos treated at very early time points (before 4 hr) and at higher DMH4 dosage resulted in delays to the onset of gastrulation and exogastrulae.

RESULTS

Vegf mRNA in *E. tribuloides* is Spatially Restricted to Two Pockets of Expression where the Future Sites of Spiculogenesis Will Form

We sought to determine the spatiotemporal dynamics of SM-specific components of VEGF signaling in *E. tribuloides* development. We isolated by PCR amplification 1100 bases of cDNA sequence for both *vegf* and *vegfr* from an *E. tribuloides* cDNA library. We confirmed the orthology of these sequences by reconstructing phylogenetic trees with published echinoderm VEGF and VEGFR amino acid sequences (see Supplementary

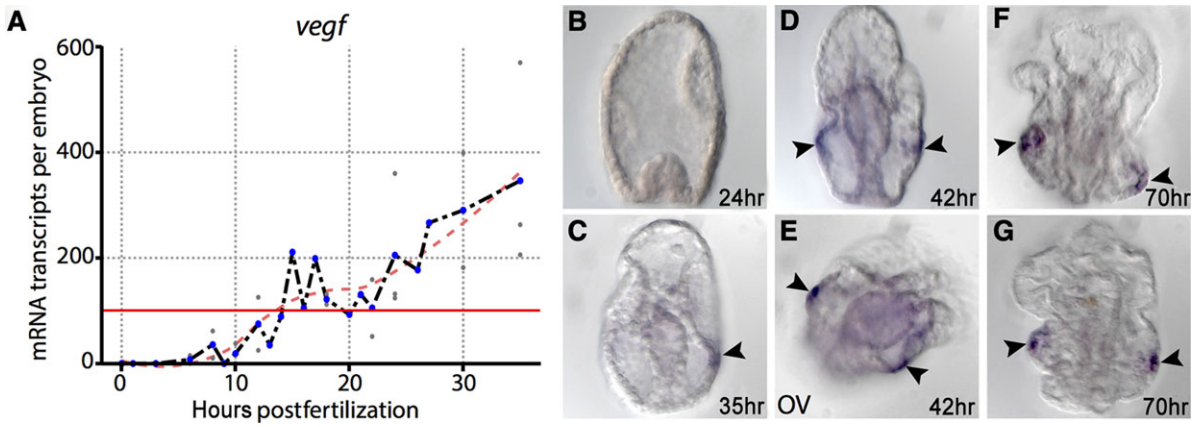

Figure 1. Temporal expression and spatial distribution of *vegf* RNA transcripts in *Eucidaris tribuloides*. (A) Quantification of *vegf* RNA transcripts per embryo by qPCR over the first 36 hr of *E. tribuloides* development. Red solid line marks 100 transcripts. Gray points represent individual measurements of individual biological replicates. Blue points represent the average of individual measurements at a particular time point. Red dashed line is a fitted moving average between all time points. Black dashed line is a straight line between the average of each time point. (B–G) Detection of *vegf* spatial distribution by whole-mount in situ hybridization. All embryos are in lateral view unless otherwise indicated. Arrows mark probe detection. OV, oblique view. (B) At 24 hpf, *vegf* transcripts are not detected. (C) At 35 hpf, *vegf* is observed near the endodermal-ectodermal boundary. (D–G) At 42 and 70 hpf, *vegf* is clearly detected in two ventrolateral regions. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 1). We designed primers for qPCR and synthesized antisense, digoxygenin-labeled RNA probes for WMISH (Erkenbrack et al., 2016). In the euechinoid *S. purpuratus*, *vegf (Sp-vegf3)* is likely downstream of the NODAL signal arising from the oral ectoderm and is spatially restricted to two ventrolateral clusters at the border of the endoderm and ectoderm by the transcription factor Not (Li et al., 2012). In the cidaroid *E. tribuloides*, *vegf* transcription, as detected by qPCR, begins coming off the baseline at mid-blastula stage waffling near the "on" threshold of 100 transcripts per embryo between 15 and 24 hpf, and steadily increasing thereafter (Fig. 1A). The variance in our data for VEGF transcripts around the onset of transcription likely reflects both the low abundance of this transcript and the sensitivity of our qPCR assay. This temporal sequence in *E. tribuloides* is similar to other NODAL-responsive regulatory genes and is 3–4 hr removed from the onset of *nodal*—similar to *chordin* (Erkenbrack, 2016). Shortly after gastrulation commenced at 24 hpf, *vegf* signaling was not detected by WMISH, which is likely due to low levels of mRNA product (Fig. 1B). As mesenchyme invades the blastocoel around 35 hpf, *vegf* is first detected by WMISH in regions that are likely to be occupied by posterior endoderm and lateral ectoderm regulatory genes (Fig. 1C). By 42 hpf, and later at 70 hpf, mRNA transcripts of *vegf* are detected in two ventrolaterally positioned clusters of expression that likely lie near the endodermal–ectodermal boundary (Figs. 1D–G). The spatial distribution of *vegf* mRNA unmistakably correlates with the location of larval spiculogenesis (Wray and McClay, '88; Erkenbrack and Davidson, 2015). These observations are highly suggestive of a conserved role for *vegf* in signaling to anteriorly positioned SM cells that migrate from the tip of the archenteron to two laterally positioned cellular clusters that will form the sites of larval spiculogenesis (Erkenbrack and Davidson, 2015).

Vegfr mRNA is Spatially Restricted to SM Cells in *E. tribuloides*

We concurrently assayed the spatiotemporal deployment of *vegfr* mRNA, the receptor of VEGF ligand. In euechinoids *vegfr* is expressed in early development exclusively in SM (Duloquin et al., 2007; Li et al., 2012) and is downstream of the SM regulatory genes *hex, alx1,* and *tbrain* (Oliveri et al., 2008; Rafiq et al., 2012). In *E. tribuloides, vegfr* comes off the baseline at mid-blastula stage and crosses the 100 transcript count near the onset of gastrulation at 22 hpf (Fig. 2A). Transcripts of *vegfr* were not detected by WMISH at the earliest gastrula stages, but were detected in a small subset of mesenchymal cells as mesenchyme ingressed into the blastocoel in increasing numbers (Fig. 2B and C). By mid-gastrula stage at 42 hpf *vegfr* is detected in a few mesenchymal cells that appear laterally and more near the blastopore (Fig. 2D and E), suggesting these cells may be showing a preference for the ventral region of the embryo. The small number of mesenchymal cells in which *vegfr* is transcribed is consistent with observations of 6–12 cells expressing SM regulatory factors in *E. tribuloides* (Wray and McClay, '88; Erkenbrack and Davidson, 2015), suggesting that *vegfr* is specifically transcribed in the *Et* SM lineage. Later, at 70 hpf, *vegfr*-positive cells are positioned

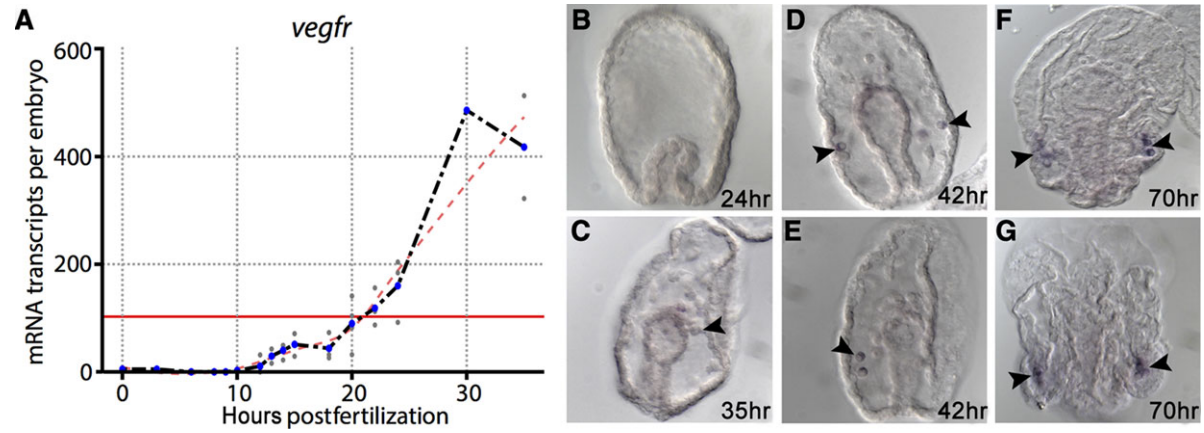

Figure 2. Temporal expression and spatial distribution of *vegfr* RNA transcripts in *Eucidaris tribuloides.* (A) Quantification of *vegfr* RNA transcripts per embryo by qPCR over the first 36 hr of *E. tribuloides* development. Red solid line marks 100 transcripts. Gray points represent individual measurements from individual biological replicates. Blue points represent the average of individual measurements at a particular time point. Red dashed line is a fitted moving average between all time points. Black dashed line is a straight line between the average of each time point. (B–G) Detection of *vegfr* spatial distribution by whole-mount in situ hybridization (WMISH). Arrows mark probe detection. (B) At 24 hpf, *vegf* is not detected by WMISH. (C) At 35 hpf, *vegfr* RNA transcripts can be detected in a subset of mesenchymal cells. (D and E) At 42 hpf, *vegfr* is clearly seen in mesenchymal cells migrating toward the ventrolateral region where spiculogenesis will later occur. (F and G) At 70 hpf, *vegfr* is spatially restricted to two clusters of cells that have congregated at the ventrolateral sites of skeletal synthesis. [Color figure can be viewed at wileyonlinelibrary.com]

in two clusters of four to six cells each within the blastocoel precisely at the embryonic location where spiculogenesis will occur (Fig. 2F and G).

A Potent Inhibitor of VEGF Signaling Disrupts Spiculogenesis in *E. tribuloides*

The foregoing results suggest that, as observed in euechinoids, VEGF signaling likely plays a role in the specification and migration of SM in *E. tribuloides* to the ventrolateral clusters. We tested this hypothesis by culturing *E. tribuloides* embryos in the presence of the VEGFR small molecule inhibitor DMH4 (Hao et al., 2010). Embryos cultured in the presence of 3 μM DMH4 at mid-blastula stage onwards did not exhibit larval spicules at 5 days postfertilization (Table 1). At lower concentrations ($\leq$1 μM), most embryos exhibited larval spicules. Additionally, specification of non-SM, for example, pigment cells, was not affected, suggesting that DMH4 specifically targets VEGFR kinase activity, as previously reported in zebrafish (Hao et al., 2010). As we neither performed WMISH on DMH4-treated embryos or interrogated their development outside of gross morphological observations, we cannot with any certainty point to whether VEGFR-inhibited embryos exhibited SM cells that failed to reach the ventrolateral clusters altogether or if SM cells migrated to their destination and subsequently failed to synthesize spicules. Importantly, while we have conducted a dose response in order to control for nonspecific inhibition of other tyrosine kinase receptors, we cannot rule out that DMH4 binds promiscuously to targets other than VEGFR kinases, for example, interacting with a BMP receptor. Future investigations could strengthen these arguments by conducting parallel perturbations with axitinib, another potent VEGF inhibitor. Lastly, it should be noted that embryos cultured in the presence of this inhibitor at early time points or at higher concentrations (>3 μM) exhibited delays in gastrulation and, in some cases, developed as exogastrulae, suggesting that either VEGFR plays a role in gastrulation, which is unlikely, or DMH4 has off-target effects in this echinoid at

Table 1. Phenotypic effect of the VEGFR pharmacological inhibitor DMH4 on *Eucidaris tribuloides* early development

	DMH4 concentration (nM)		
Observation (time scored)	300	1,000	3,000
Gastrulation delay (24 hpf)	0/19	2/17	3/21
Exogastrula (36 hpf)	0/20	1/22	1/19
Pigment cells (72 hpf)	18/18	18/18	20/20
Larval spicules (120 hpf)	21/21	14/21	0/24

higher doses. With these caveats in mind, the scored phenotype here in the presence or absence of larval spicules in *E. tribuloides* embryos cultured in the presence of a potent VEGFR-antagonist suggests that the VEGF signaling pathway plays a conserved role in specification of the eleutherozoan embryonic SM cell type.

DISCUSSION

In the preceding sections, we describe observations and experimental data that implicate VEGF signaling in specification and migration of SM of the cidaroid sea urchin *E. tribuloides*. Our data fill an important gap in the developmental evolution of echinoderm skeletogenesis and add to a growing list of observational and experimental data on the SM cell type, which now includes at least 12 echinoderm taxa (Koga et al., 2014, 2016; McIntyre et al., 2014; Minokawa, 2016). Skeletogenesis in echinoderms provides an auspicious system to probe the evolution and appearance of novel structures in early development (Koga et al., 2014; Minokawa, 2016), as well as an experimental platform with evolutionary replicates to interrogate the evolution of cell-type identity and the GRNs that specify them (Arendt et al., 2016). Below we elaborate on these concepts, placing our results in an evolutionary context that paints an important, heretofore undescribed segment of the larger canvas depicting the developmental evolution of the SM cell type in echinoderms.

Specification and Interaction of SM in *E. tribuloides*

Our data here suggest that VEGF signaling exhibits a conserved role in spiculogenesis. In unperturbed embryos of *E. tribuloides,* SM positions itself in a markedly similar orientation despite the distinct embryonic landscape that these cells traverse. Thus, the mechanisms that give rise to the correct spatial positioning of this homologous cell lineage are likely conserved in echinoids. An interesting future research direction would be to investigate the molecular underpinnings of ectodermal–mesenchymal interactions at the site of spiculogenesis. While our molecular and experimental observations here present a preliminary analysis of this system in a heretofore data-poor echinoderm clade, future studies could extend them by performing double WMISH on SM specification genes coupled with targeted perturbations of VEGF signaling.

Migration of SM in *E. tribuloides*

Initial observations of *E. tribuloides* SM, as well as other mesenchymal cell types, utilized time lapse video to trace mesenchymal migration from the tip of the archenteron throughout the blastocoel (Wray and McClay, '88). These authors concluded that SM cells in this echinoid initially move randomly after ingression and later become restricted to directional movement toward the sites where spiculogenesis will occur (Fig. 3A) (Wray and McClay, '88). Our results offer an in principle explanation as to why this initial seemingly random movement occurs. Transcription of *vegf* RNA—the protein product of which is a ligand that interacts with its receptor (VEGFR) in SM—does not begin in earnest until 24 hpf (Fig. 1A). SM ingression in *E. tribuloides* occurs around 28 hpf, approximately when the archenteron has extended about one-third of the way into the blastocoel. Thus, if SM requires stimulation of VEGF receptor to alter gene expression, and thereby their future migratory behavior, it stands to reason that at the time of their ingression little to no VEGF ligand is in the blastocoel to induce the behavioral change. Thus, the random, directionless movement initially seen in *E. tribuloides* SM may be explained by the duration of ligand production and secretion, as well as its subsequent diffusion into a blastocoel filled with sulfated macromolecules that may play a role in SM migration (Katow and Solursh, '81; Solursh and Katow, '82; Fujita et al., 2010; Piacentino et al., 2016). An experimental hypothesis motivated by these observations is that overexpression of *vegf* RNA in this echinoid should diminish the observed random movement. This line of argumentation begs the question as to whether the discrepancy observed between the onset of *vegf* and *vegfr* transcription and the developmental address, as well as the timing, of mesenchymal ingression can inform our understanding of the evolution of the echinoderm SM cell type.

Equal Distribution of Migratory *E. tribuloides* SM to Two Ventrolateral Clusters

Migration of SM in *E. tribuloides* presents us with an interesting conundrum of developmental mechanism when viewed in the context of SM cell types in other echinoderms. In euechinoids and ophiuroids, SM ingresses into the blastocoel prior to gastrulation at the base of the vegetal pole, where they are in contact with the basal lamina and are proximal to the future sites of VEGF ligand-secreting cells and the ventrolateral clusters (McIntyre et al., 2014). This spatial arrangement of SM ingression in euechinoids and ophiuroids increases the likelihood that each pocket of VEGF expression will come to be occupied by SM. In *E. tribuloides*, SM ingresses near the equatorial center of the blastocoel and must traverse an embryonic distance of at least 50 μm, migrating to one of two bilateral sites where spiculogenesis will occur. Relative to euechinoids and ophiuroids, the developmental address of SM ingression in cidaroids presents an interesting problem for their embryos: nondirectional movement could lead to all or most SM cells migrating toward one VEGF expressing domain over the other, since the previously mentioned phenomenon of randomly migrating SM in *E. tribuloides* suggests that effectuating signal does not alter SM behavior at the time of mesenchymal ingression. This scenario begs the question as to whether there exists a mechanism in cidaroids, as well as in echinoids as a clade, that ensures distribution of SM to both VEGF-secreting domains. While we have not tested this, we put forward two hypotheses for future research efforts. One hypothesis is that the spatial distribution and positioning of SM cells at the tip of the archenteron will determine to which ventrolateral cluster each cell with migrate (Fig. 3B). However, as

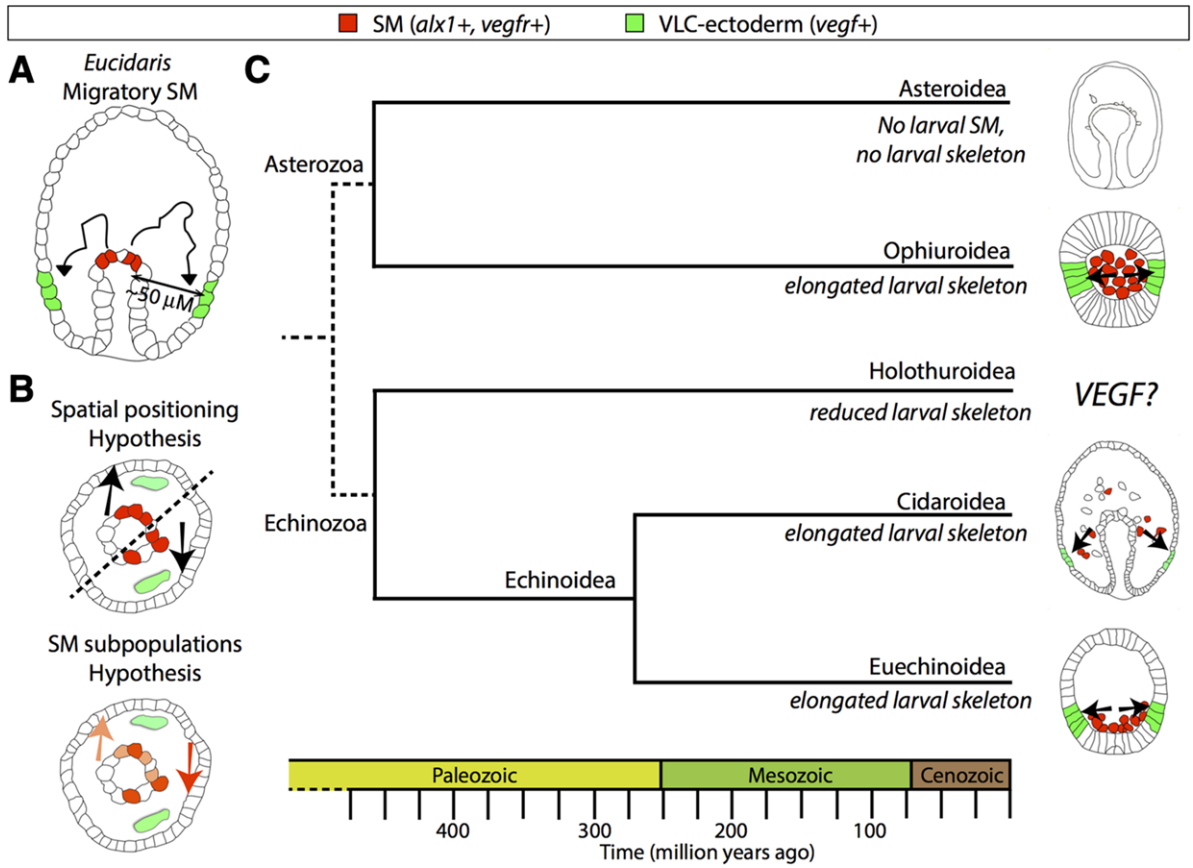

Figure 3. Migratory skeletogenic mesenchyme in *Eucidaris tribuloides* and evolution of homologous skeletogenic cell types in other echinoderms. (A) In *E. tribuloides*, skeletogenic mesenchyme ingresses at the anterior end of the archenteron after gastrulation has begun. These cells then migrate from the archenteron first randomly and later ventrolaterally toward the two future sites of spiculogenesis, a distance of at least 50 μm. (B) Two proposed hypotheses that could explain the bilateral migration of SM cells to the two ventrolateral clusters in *E. tribuloides*. The spatial positioning hypothesis posits that the dispersed spatial distribution of SM cells residing near the tip of the archenteron give rise to SM that are in different proximity to each VEGF signal. The proximity to each signal will determine to which ventrolateral cluster each SM cell migrates. The SM subpopulation hypothesis posits that during or after ingression two populations of SM cells exist. Each SM subpopulation has distinct regulatory states that then determine the behavior and migration of each SM cell. (C) A phylogeny of the eleutherozoan echinoderms and two subclasses of echinoids showing their embryos, larval skeleton type, and their estimated times divergence times in deep time (Pisani et al. 2014; Thompson et al. 2015). Echinoids and ophiuroids researched thus far that exhibit an elongated larval skeleton possess SM cells that ingress proximal to VEGF-secreting cells near the vegetal plate with the exception of the sister clade to euechinoids, the cidaroids. In holothuroids whether VEGF signaling plays a role in spiculogenesis is not clear and has been indicated. Green cells, *vegf*+ ventrolateral clusters; Red (or orange) cells, *vegfr*+/*alx1*+ skeletogenic mesenchyme (SM); arrows indicate direction of SM migration. VLC, ventrolateral clusters. [Color figure can be viewed at wileyonlinelibrary.com]

was previously shown, initial migration of SM in this echinoid is seemingly random, and this hypothesis assumes that ingressing SM will behave according to their spatial location in the archenteron. An alternative scenario is what we call the SM subpopulations hypothesis (Fig. 3B). In this case, either at the time of ingression or shortly thereafter, the regulatory states are distinct in half of the SM cells, and these different regulatory states ensure that half of the SM migrates to one VEGF expression domain and vice versa. An additional signal is the most likely candidate mechanism that could alter regulatory states in SM. But even in the case of a second signal, this would still likely involve spatial positioning at the time of SM ingression. These

hypotheses highlight the need for further exploration into the developmental mechanisms underlying this cellular migration.

Ancestral State Reconstruction of SM in Echinozoans and Eleutherozoans

Perhaps more fascinating is reconstructing the ancestral states that likely existed in the most recent common ancestor (MRCA) of eleutherozoans (Erkenbrack et al., 2016). First, considering only the echinoids, these two clades shared a common ancestor at least 268 million years ago (Thompson et al., 2015), and it is highly probable that the MRCA utilized VEGF signaling to specify and direct its SM lineage to the ventrolateral clusters. Equally intriguing, the observation that ophiuroids utilize VEGF to specify migratory SM (Morino et al., 2012) as well suggests that the GRN modules encoding migration of SM are downstream of and are tightly coupled to the initial skeletogenic specification modules. These observations support the hypothesis that the adult skeletogenic program was hijacked and installed into the early embryo (Gao and Davidson, 2008) and further suggest that these GRN modules are co-opted jointly. Furthermore, the implications for adult skeletogenesis are even now more clear, as these data from early developmental skeletogenic specification predict that VEGF signaling should be of considerable importance for the adult echinoderm skeletogenic program. This has been evidenced by studies in *E. tribuloides* (Gao and Thompson et al., 2015) and can be made clear by additional future studies on the genes and signaling systems directing skeletogenic plate formation in echinoderm juveniles.

Evolution of SM Cell Types in Echinoderms

In the SM cell type of echinoderms, developmental evolutionary biologists are presented with a comparative platform to investigate the evolution of novelty and evolutionary rewiring of developmental GRNs. In euechinoids, SM cell types run the gamut of developmental variation. For instance, in indirect developing embryos SM is specified very early in development, where as in direct developing embryos they are specified sometime later (Wray and Raff, '89). There are distinct differences in the total number and size of SM cells produced in numerous euechinoid lineages (Wray and McClay, '88; Yajima, 2007), as well as the existence of alternative regulatory states in different euechinoid taxa (Yamazaki and Minokawa, 2015). In the sister subclass of euechinoids, cidaroids, we observe a shift in timing that is accompanied consequently by a spatially distinct developmental address of SM ingression, as well as alternative regulatory states (Yamazaki et al., 2014; Erkenbrack and Davidson, 2015; Erkenbrack et al., 2016). And despite this vast developmental variation it is important to recognize that these cell types likely descended from a common ancestral echinoid SM cell type. As the numerous lineages of echinoids diverged over evolutionary time, alternative GRN modules may have been co-opted to run with or downstream of the ancestral SM cell type program, potentially changing the phenotype, absolute number of SM cells, or the regulatory state. Investigation of these alterations will inform our understanding of the evolvability of SM cell types and their underlying GRNs. Thus, comparative analyses of echinoid SM cell types are rife for investigation at the genomic level.

Even further back in evolutionary time holothuroids and ophiuroids present—in the case of the former—a potentially exemplary case of degeneration of a markedly reduced developmental structure and its GRN (Hart, 2002; McCauley et al., 2012) and—in the case of the latter—remarkable convergent evolution of the larval skeleton (Strathmann, '88; Littlewood et al., '97). Thus, developmental studies on echinoderm skeletogenesis have the potential to inform hypotheses that aim to reveal the evolutionary relationships among the eleutherozoan echinoderms (Smith, '84; Pisani et al., 2012; Telford et al., 2014). Since both echinoids and ophiuroids exhibit a pluteus larval form that is either a product of parallel evolution, that is, convergent, or existed in a common ancestor, the SM cell type and their resulting larval structures have an important role to play in this conversation since the skeletogenic structure is the lynchpin of the pluteus larva. Studies that expand our taxonomic developmental data set, such as the recent study suggesting that regulatory gene orthologs in echinoids and ophiuroids are similarly deployed in the synthesis of pluteus larval skeletal structures (Morino et al., 2016), have potential to inform these debates. Data presented here add a new wrinkle to developmental evolutionary studies of skeletogenic specification in echinoids and highlight additional developmental variations in the long and winding evolutionary history of the extraordinary SM cell type of echinoderms.

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